ISOLATION AND CHARACTERIZATION OF BACTERIA THAT DEGRADE NITROGLYCERIN

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ABSTRACT

Six strains of bacteria capable of degrading nitroglycerin (NG) were isolated from soil previously exposed to NG. Nitroglycerin degradation by each of the strains was characterized, with representative degradation patterns reported for two strains, *Pseudomonas putida* I-B and *P. putida* II-C. It was found that *P. putida* I-B denitrated NG into isomers of MNG with a preference for the denitration of nitroglycerin at the C2 position. *P. putida* II-C denitrated NG in a random fashion, producing a mixture of DNG isomers and a mixture of MNG isomers. Both strains liberated nitrite as a product of NG denitration. All six isolates were tested for their abilities to tolerate up to 3.96 mM NG. Nitroglycerin concentrations up to 1.32 mM did not inhibit growth of the six NG-degrading isolates, whereas the growth of *P. syringae* pathovar *syringae*, an organism not previously exposed to NG, was inhibited at 0.44 mM NG. The growth of one NG-degrading isolate was uninhibited at 3.52 mM NG and only partially inhibited at 3.96 mM NG.

INTRODUCTION

Nitroglycerin (NG), or glycerol trinitrate, is one of the most widely used explosives. It is the main component of dynamite and it serves as a plasticizer for nitrocellulose in smokeless, doublebase propellants (1, 2). Medicinally, nitroglycerin is a vasodilator, and has been used for the treatment of angina pectoris (1).

The discharge of process water from nitroglycerin manufacture poses an environmental threat, especially to aquatic ecosystems. It has been estimated that a full production nitroglycerin manufacturing plant releases 35.3 mg of NG per liter of waste water, discharging over 166,000 liters of waste water daily (2). With a solubility of 1.8 g l^{-1} in water at 20 °C (1), nitroglycerin is easily transported throughout the environment. Biological impact studies of ponds that received munitions-manufacturing waste water at the Badger Army Ammunition Plant (BAAP), in Baraboo, WI, revealed that nitroglycerin manufacturing wastes exerted toxic effects on algal and macroinvertebrate populations. The algal species identified at NG-contaminated study sites were characterized as pollution tolerant, and populations of benthic macroinvertebrates were absent, both indicating toxic environmental conditions (3).

Whereas nitroglycerin exerts toxic effects on organisms ranging from bacteria to mammals, various studies have demonstrated that NG is amenable to biological breakdown (2). Because NG has been administered to humans for medicinal purposes, its physiological effects were originally studied in mammals. By orally administering ^{14}C-labeled nitroglycerin to rats, it was determined that NG was metabolized via two pathways. The first pathway consisted of the stepwise denitration of NG to dinitroglycerin (DNG), mononitroglycerin (MNG), and glycerol, which was ultimately oxidized to CO_2. Through a second pathway, denitration products were conjugated to glucuronic acid (4). In an *in vitro* study, in which rat liver homogenates were incubated with ^{14}C-labeled nitroglycerin, the enzyme glutathione-organic nitrate reductase was implicated in the stepwise denitration of NG (5). Partially purified preparations of this rat liver enzyme denitrated nitroglycerin in the presence of reduced glutathione. One molecule of NG reacted with two

glutathione molecules to release an inorganic nitrite ion from either the C2 or the C3 position of nitroglycerin, forming 1,3-DNG or 1,2-DNG, respectively (6). In another *in vitro* study, it was found that the plasma membrane fraction of bovine coronary smooth muscle cells enzymatically denitrated NG, with a concurrent release of nitric oxide (7).

More recently, work has been conducted to characterize the NG-degradation ability of the white-rot basidiomycete, *Phanerochaete chrysosporium*. Servant *et al.* (8) demonstrated *in vivo* that NG was aerobically degraded into DNG and MNG, with the liberation of nitrite ions and nitric oxide. Upon release, a fraction of the nitrite was rapidly oxidized to nitrate, while the remaining nitrite was reduced to nitric oxide and detected as complexes with non-heme iron-sulfur centers. These nitric oxide complexes were the same as those normally formed during nitrite metabolism by *P. chrysosporium*. Nitric oxide was also detected as a complex with a reduced heme, possibly as a direct NG-denitration product released through an as yet uncharacterized pathway. Because nitrate ions were not directly released through NG denitration, the authors hypothesized that denitration occurred via a reduction reaction catalyzed by a glutathione S-transferase-type system, similar to that previously characterized in mammalian systems, rather than by an esterase reaction.

To further characterize fungal nitroglycerin metabolism, *in vitro* NG degradation by crude cell extracts and by subcellular fractions of *P. chrysosporium* was studied (9). Based on these studies, 30 percent of the cytosolic NG-denitration activity was attributed to a glutathione S-transferase system. Additional anaerobic, NADPH-dependent, reductive nitrogycerin denitration, found in cytosolic and microsomal subcellular fractions, was believed to be a result of cytochrome-P450 activity. Microsomal subcellular fractions were also noted to contain anaerobic, Fe^{2+}-dependent NG-denitration activity, surmised to be related to an additional hemoprotein enzyme system. This Fe^{2+}-dependent NG degradation system released nitric oxide as an NG-denitration product. Thus, multiple enzymatic systems are involved in the denitration of nitroglycerin by *P. chrysosporium*. Further, the observation that the fungus *Geotrichum candidum* cleaved nitrite groups from the NG molecule with a regioselectivity different from that of *P. chrysosporium* (10, 11) suggests that other, yet uncharacterized NG-degradation pathways may exist among additional species of fungi.

Several studies have been conducted to investigate the ability of bacteria to degrade nitroglycerin. In an initial study, Wendt *et al.* (12) found that both mixed and pure bacterial cultures, obtained from domestic sewage activated sludge, were capable of degrading NG in a stepwise fashion, via the di- and monoesters. Cultures were unable to utilize NG as a sole carbon source, and no attempts were made to identify the organisms involved or to characterize enzymatic pathways. A follow-up study (13), in which a mixed bacterial culture was assayed for its ability to degrade nitroglycerin, confirmed the findings of Wendt *et al.* (12), that bacteria were unable to utilize NG as a sole carbon source. More recently, NG-degrading strains of *Bacillus thuringiensis/cereus*, *Enterobacter agglomerans* (14), and *Agrobacterium radiobacter* (15) have been identified.

A primary goal of our laboratory is to provide a detailed characterization of bacterial enzyme systems used to degrade nitroglycerin. Toward this goal, we discuss the *in vivo* characterization of six strains of NG-degrading bacteria. Attention is focused on two isolates, one which denitrates NG preferentially into 1,3-DNG and 1-MNG, and another which randomly denitrates NG into a mixture of DNG and MNG isomers. In addition, the culture densities attained by these isolates in medium containing 0-3.96 mM nitroglycerin are compared.

METHODS

Materials: Nitroglycerin, dissolved in water at concentrations of 4.40 mM and 6.60 mM, was obtained from Olin Corporation. Analytical reference standards of NG, 1,2-DNG, 1,3-DNG, 1-MNG, and 2-MNG were purchased from Radian. Spizizen minimal (SM) phosphate salt medium (16), adjusted to pH 7.0, was used for the cultivation of all strains. As noted, various concentrations of nitroglycerin were added to SM medium, and $(NH_4)_2SO_4$ was omitted from the medium as indicated. All incubations were conducted aerobically, at 30 °C. Aerobic liquid

cultures were shaken at 250 RPM in a New Brunswick water bath shaker. Optical densities of cultures were determined with a Klett-Summerson photoelectric colorimeter, using a red filter.

Isolation of nitroglycerin-degrading bacteria: Soil samples were collected at BAAP from sites previously contaminated through nitroglycerin manufacturing processes. Bacteria capable of degrading nitroglycerin were isolated based on two characteristics: their ability to utilize NG as a sole source of nitrogen and their ability to tolerate NG concentrations inhibitory to the growth of most bacteria.

For the isolation of bacteria capable of utilizing NG as a nitrogen source, soil samples were used to inoculate liquid SM medium lacking $(NH_4)_2SO_4$ but supplemented with 0.44 mM NG. An enrichment technique was used to isolate bacteria capable of tolerating NG concentrations inhibitory to other organisms. SM-medium starter cultures, inoculated with soil, were used to successively inoculate a series of SM cultures containing the following increasing amounts of nitroglycerin: 0.13 mM, 0.22 mM, 0.44 mM, 0.88 mM, 1.10 mM, 1.32 mM, and 2.20 mM. The isolated organisms were then assayed for their abilities to degrade NG.

Identification of nitroglycerin-degrading bacteria: NG-degrading bacteria isolated at BAAP were identified based on morphological observations, the API 20E identification system for gram negative bacteria (bioMerieux), as well as additional physiological tests.

Cultivation conditions used to evaluate NG degradation by the BAAP isolates: Starter cultures (SM medium supplemented with 1.32 mM nitroglycerin) were used to inoculate triplicate SM-medium cultures, supplemented with 1.32 mM NG, to 10 Klett Units (K.U.). At recorded intervals, the optical density of each culture was measured, and a sample was removed for NG-denitration analysis. Cells were removed from samples by centrifugation and the supernatant fluid was stored at -80 °C until analyzed by high-performance liquid chromatography (HPLC).

Nitroglycerin toxicity: Each BAAP strain (SM-medium starter culture grown without NG) was inoculated to 10 K.U. into a duplicate series of flasks containing SM medium supplemented with one of the following nitroglycerin concentrations: 0 mM, 0.44 mM, 0.88 mM, 1.32 mM, 1.76 mM, 2.20 mM, 2.64 mM, 3.08 mM, 3.52 mM, and 3.96 mM. The cultures were incubated for 24 hours, at which point, turbidity was measured.

Analytical methods: Samples were analyzed for the presence of NG, 1,3-DNG, 1,2-DNG, 2-MNG, and 1-MNG by HPLC (Beckman). The UV detector was set at 204 nm. Samples were injected onto a 4 µm Nova-Pak radial compression C_{18} column (length, 100 mm; diameter, 5 mm [Waters]) and eluted with a methanol-water step gradient, at a flow rate of 1.25 ml/min (17, 18). Isomers of MNG were resolved in a mobile phase consisting of five percent methanol, isomers of DNG were resolved in 30 percent methanol, and NG was eluted in 65 percent methanol. The analytical column was protected by a 0.5 µm precolumn filter (Upchurch Scientific) and a 5 µm ODS Safeguard guard cartridge (MetaChem Technologies).

Samples were analyzed for the presence of nitrate and nitrite by ion chromatography. A Dionex DX-100 Ion Chromatograph with conductivity detector was used. Samples were injected onto an AS4A Dionex IonPac column (length, 250 mm; diameter, 4 mm), protected by a Dionex AG4A guard column (4 mm). The mobile phase consisted of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate, run at a flow rate of 2 ml/min.

RESULTS

Strain isolation and identification: Six different strains of NG-degrading bacteria, based on colony morphology, were isolated and chosen for additional study. These strains were designated

I-A, II-A, I-B, II-B, I-C, and II-C. Two strains (I-B and I-C) were isolated based on their abilities to utilize NG as a nitrogen source, while the other four strains were isolated through enrichment procedures. All six strains were gram negative rods. Strain I-A was identified as *Klebsiella oxytoca*, strains II-A and I-C were identified as *Pseudomonas fluorescens*, and strains I-B, II-B, and II-C were identified as *P. putida*.

NG degradation by the BAAP isolates: Time course experiments were used to characterize pathways by which the six BAAP isolates degraded nitroglycerin. By omitting glucose from SM medium containing nitroglycerin, it was determined that none of the isolates would grow when provided with NG as the sole carbon source. In the absence of $(NH_4)_2SO_4$, all isolates were able to utilize nitroglycerin as a nitrogen source, however growth rates and final cell yields increased with the inclusion of $(NH_4)_2SO_4$ (data not shown). Further, cultures of *P. putida* strains I-B and II-C lacking $(NH_4)_2SO_4$ took twice as long to denitrate 1.32 mM nitroglycerin as cultures provided with $(NH_4)_2SO_4$ (data not shown). Fig. 1 shows the denitration of 1.32 mM nitroglycerin and its intermediates, over 24 hours, by *P. putida* strains I-B and II-C. Of note is the observation that NG-denitration enzymes used by these two strains removed nitrite groups from nitroglycerin with differing regioselectivities. *P. putida* I-B regioselectively denitrated nitroglycerin into 1,3-DNG and 1-MNG (Fig. 1a), while *P. putida* II-C (Fig. 1b) denitrated NG into a mixture of 1,3- and 1,2-DNG and a mixture of 2- and 1-MNG. More specifically, *P. putida* I-B denitrated NG into 1,3-DNG/1,2-DNG with a ratio of 7.6, whereas *P. putida* II-C denitrated NG into 1,3-DNG/1,2-DNG with a ratio of 0.5. *K. oxytoca* I-A and *P. fluorescens* strains II-A and I-C denitrated NG regioselectively, favoring removal of nitrite from the C2 position of nitroglycerin, while *P. putida* II-B denitrated NG in a non-regioselective fashion (data not shown).

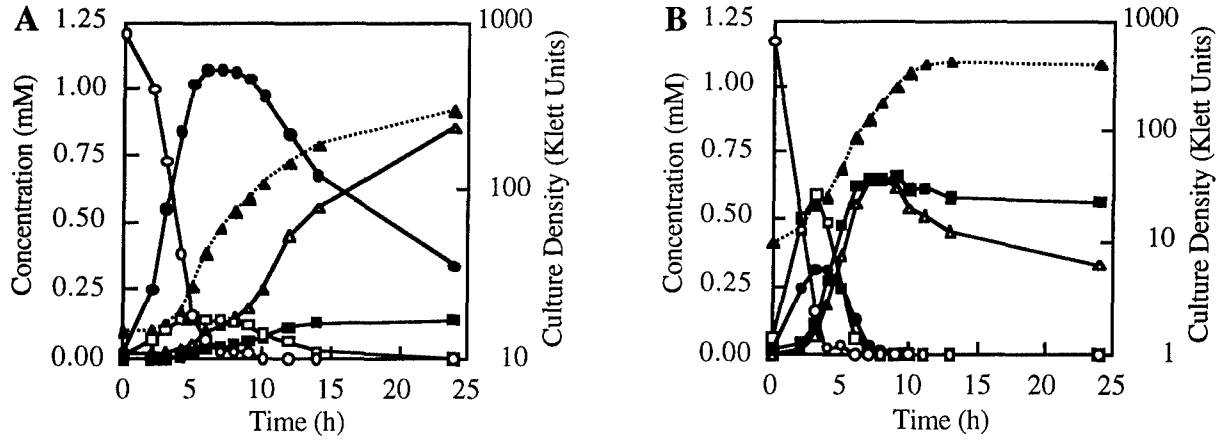

Figure 1. Time course of NG degradation and culture growth by (a) *P. putida* I-B and by (b) *P. putida* II-C. NG was provided at an initial concentration of 1.32 mM. Values are the averages of three independent experiments. O, NG; ●, 1,3-DNG; □, 1,2-DNG; ■, 2-MNG; △, 1-MNG; ▲, culture density.

Analyses were conducted to determine whether *P. putida* strains I-B and II-C liberated nitrate or nitrite as a product of nitroglycerin denitration. These experiments demonstrated that both strains liberated nitrite (Fig. 2). For both strains, the concentrations of nitrite that accumulated were approximately stoichiometric to the amounts of NG degraded and to the amounts of NG-degradation intermediates remaining in the medium. Significant amounts of nitrate were not

detected in the growth medium of either strain. Through control experiments, in which *P. putida* strains I-B and II-C were grown in SM medium supplemented with 2.64 mM $NaNO_3$ or $NaNO_2$, it was confirmed that neither strain reduced nitrate to nitrite or oxidized nitrite to nitrate, under the routinely employed culture conditions.

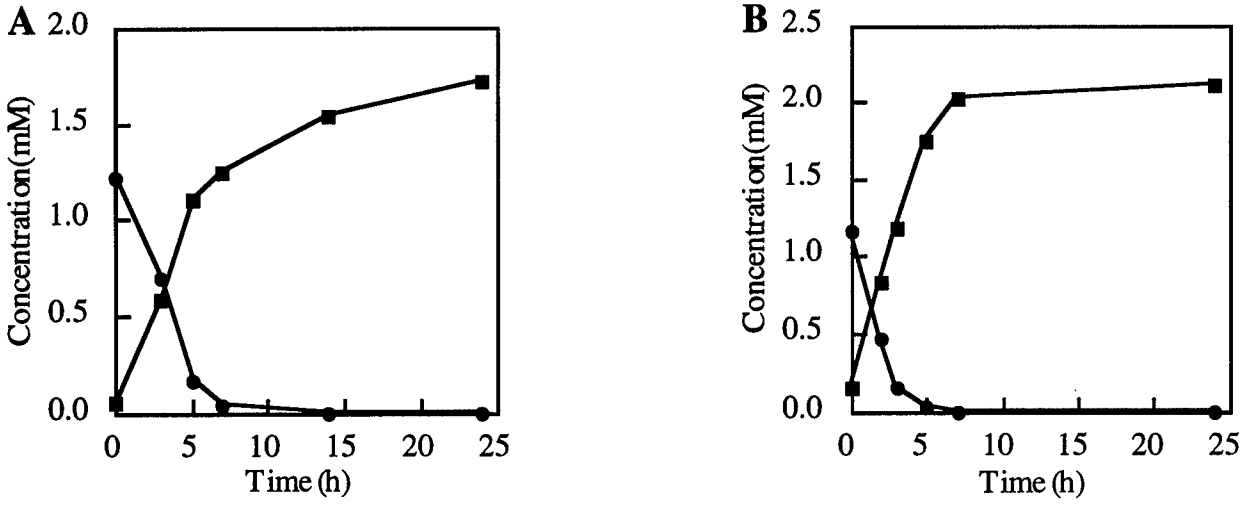

Figure 2. Time course of NG degradation and nitrite accumulation by cultures of (a) *P. putida* I-B and by cultures of (b) *P. putida* II-C. Values are the averages of three independent experiments. ●, NG; ■, nitrite.

To verify that NG-breakdown was enzymatic, rather than a result of spontaneous degradation, cell-free control reactions were incubated under normal culture conditions without a notable decrease in nitroglycerin concentration. Heat-killed cells, incubated for 24 hours in SM medium supplemented with 0.88 mM NG, caused no significant decrease in culture-medium nitroglycerin concentrations. This demonstrated that NG did not adhere to the surface of non-viable cells. In addition, nitroglycerin was not degraded by medium from NG-degrading cultures after passage through a 0.45 μm filter, implying that extracellular enzymes are not involved in this process.

Nitroglycerin toxicity: For this work, we defined nitroglycerin toxicity as the inhibition of bacterial growth as measured by culture turbidity. The nitroglycerin-degrading bacteria isolated at BAAP exhibited varying degrees of tolerance to NG (Fig. 3). Cultures of *K. oxytoca* I-A, the most NG-tolerant strain, grew to a density of 204 K.U. in the presence of 3.96 mM nitroglycerin. *P. putida* II-C grew to a density of 330 K.U. in the presence of 3.08 mM nitroglycerin, whereas turbidities of *P. putida* I-B cultures decreased in the presence of greater than 1.32 mM NG. Depending on the strain, the six BAAP isolates grew uninhibited in cultures containing from 1.32 mM to 3.52 mM NG. However, the growth of *P. syringae* pathovar *syringae,* a bacterium presumably not previously exposed to NG, was inhibited by 0.44 mM nitroglycerin.

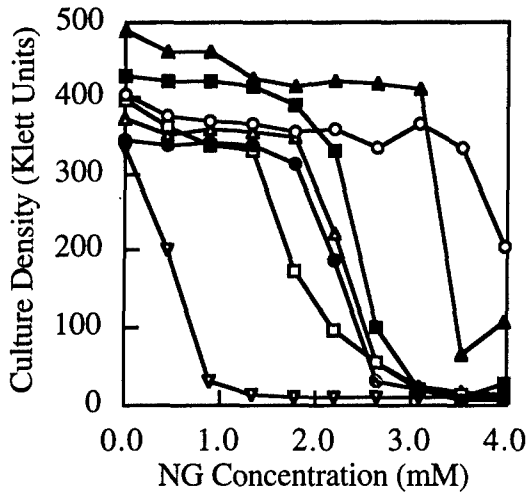

Figure 3. Culture densities attained by bacteria isolated at BAAP after 24 hours of incubation in the presence of NG. Each culture density reading is the average of two independent experiments. O, *K. oxytoca* I-A; ●, *P. fluorescens* II-A; □, *P. putida* I-B; ■, *P. putida* II-B; Δ, *P. fluorescens* I-C; ▲, *P. putida* II-C; ▼, *P. syringae* pathovar *syringae*.

CONCLUSIONS

Through our efforts to isolate and characterize nitroglycerin-degrading bacteria, we have noted that different strains of bacteria denitrate NG with varying regioselectivities. Nitroglycerin is comprised of a three-carbon backbone, with nitrate groups bonded to the C1, C2, and C3 positions. As a consequence of this structure, 1,2-dinitroglycerin can be formed by denitration at the C1 or the C3 positions, while 1,3-dinitroglycerin can only be formed by denitration at the C2 position. If enzymes catalyzing the removal of nitrite lacked regioselectivity, cleavage would occur randomly at all three positions, forming 1,3-DNG/1,2-DNG with a ratio of 0.5 (19). As reported, NG-denitration enzymes employed by *P. putida* I-B exhibited regioselectivity, preferentially denitrating nitroglycerin at the C2 position. Conversely, *P. putida* II-C randomly denitrated nitroglycerin. The different regioselectivities of these two strains suggests that among bacteria, different enzymes may be employed in the denitration of nitroglycerin.

As shown in the NG-denitration profiles for *P. putida* strains I-B and II-C presented in Fig. 1, these strains required different amounts of time to denitrate nitroglycerin to mononitroglycerin. *P. putida* II-C degraded almost all of the nitroglycerin present in its culture medium to isomers of DNG and MNG within four hours. Both isomers of dinitroglycerin, generated by NG degradation, were denitrated into MNG within approximately eight hours. Curiously, NG was completely degraded into MNG before the organism fully entered the exponential phase of cellular growth. Within a similar time frame, *P. putida* I-B also denitrated NG into DNG before entering exponential growth. However, *P. putida* I-B did not denitrate DNG with the same efficiency as strain II-C. It is reported that isomers of dinitroglycerin exert as significant a toxicity on mice and rats as nitroglycerin (2). Assuming that dinitroglycerin also exerts a toxic effect on bacteria, *P. putida* I-B may not have achieved a high cell density as rapidly as *P. putida* II-C because it was not as effective in removing toxic DNG from its culture medium. While not all of the BAAP isolates degraded NG as efficiently as *P. putida* II-C, all six strains denitrated NG into DNG before they entered exponential growth (data not shown). In addition, based on the observation that nitroglycerin was denitrated more slowly in the absence on an alternate nitrogen source, it is likely that NG is not degraded as a

preferred source of carbon or nitrogen. Instead, NG degradation may be a detoxification response by the bacteria. This hypothesis is further supported by the observation that a bacterial strain inoculated into NG-containing medium undergoes a longer lag phase, during which NG is degraded, than the same strain inoculated into medium lacking nitroglycerin. The longer lag phase may be related to time required to degrade/detoxify nitroglycerin. Thus, rather than using nitroglycerin as a preferred growth substrate, the BAAP isolates may degrade NG to detoxify their environment, which then allows them to multiply if the nutritional conditions are appropriate.

The results of nitroglycerin toxicity experiments suggest that NG-degradation enzymes of the BAAP isolates may have adapted to degrade/detoxify elevated nitroglycerin concentrations. As shown in Fig. 3, all of the BAAP isolates, with the exception of *P. putida* I-B, attained culture densities of at least 315 K.U. in the presence of 1.76 mM NG. Conversely, *P. syringae* pathovar *syringae*, an organism that had not been previously exposed to NG, exhibited an inhibition of growth in the presence of 0.44 mM NG. In addition, we observed that fourteen laboratory strains of bacteria, presumably not previously exposed to NG, were able to degrade 0.44 mM nitroglycerin to varying extents (data not shown). However, growth of these strains was inhibited by NG concentrations greater than 0.44 mM. Although the ability to degrade nitroglycerin appears to be a relatively common bacterial trait, organisms routinely exposed to nitroglycerin may have adapted to better degrade/detoxify higher concentrations of NG than organisms not regularly encountering this compound.

From an evolutionary perspective, nitroglycerin is a recent soil contaminant at the Badger Army Ammunition Plant. Although NG has only been present in the environment at BAAP for approximately fifty years, bacteria capable of degrading nitroglycerin more efficiently than those not previously exposed to this compound were readily isolated from this site. Additional studies will be required to provide detailed descriptions of the mechanisms used by the BAAP isolates to denitrate nitroglycerin.

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